Collagen-Targeting Vascular Endothelial Growth Factor Improves Cardiac Performance After Myocardial Infarction

Jing Zhang, Liang Ding, Yannan Zhao, Wenjie Sun, Bing Chen, Hang Lin, Xia Wang, Lujie Zhang, Biao Xu and Jianwu Dai

_Circulation_ 2009;119;1776-1784; originally published online Mar 23, 2009;
DOI: 10.1161/CIRCULATIONAHA.108.800565

Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2009 American Heart Association. All rights reserved. Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/cgi/content/full/119/13/1776
Collagen-Targeting Vascular Endothelial Growth Factor Improves Cardiac Performance After Myocardial Infarction

Jing Zhang, BS*; Liang Ding, MD*; Yannan Zhao, BS*; Wenjie Sun, MS; Bing Chen, PhD; Hang Lin, PhD; Xia Wang, MS; Lujie Zhang, MD; Biao Xu, MD, PhD; Jianwu Dai, PhD

Background—Vascular endothelial growth factor (VEGF) is an important active protein for the induction of angiogenesis and improvement in cardiac function after myocardial ischemia; however, the lack of a delivery system targeted to the injured myocardium reduces the local therapeutic efficacy of VEGF and increases its possible adverse effects.

Methods and Results—We produced a fusion protein (CBD-VEGF) consisting of VEGF and a collagen-binding domain (CBD). The fusion protein specifically bound to type I collagen in vitro. In addition, CBD-VEGF promoted human umbilical vein endothelial cell proliferation after binding to collagen, which indicates that it retained both growth factor activity and collagen-binding ability. When implanted subcutaneously in rats, collagen membranes loaded with CBD-VEGF were significantly vascularized. After it was injected into rats with acute myocardial infarction, CBD-VEGF was largely retained in the cardiac extracellular matrix, in which collagen I was rich. Four weeks after VEGF or CBD-VEGF was injected into the infarct border zone, cardiac function detected by echocardiography and hemodynamics was preserved in the CBD-VEGF group. Administration of CBD-VEGF also induced reduction of scar size, whereas native VEGF did not have these effects. In addition, a significant increase in the number of capillary vessels in infarcted hearts was found in the CBD-VEGF group.

Conclusions—The injection of CBD-VEGF improved cardiac function in rats with induced acute myocardial infarction. This could potentially provide a new treatment option for myocardial infarction. (Circulation. 2009;119:1776-1784.)

Key Words: vascular endothelial growth factors, collagen, angiogenesis, myocardial infarction

In recent years, ischemic heart diseases have become a global health concern. They are usually caused by stenosis or blockade of blood flow, and the common therapy is restoration of blood supply to the myocardium through angioplasty or bypass surgery. The identification of angiogenic growth factors makes molecular therapy a potential new approach to induce blood vessel formation after myocardial ischemia. Among the angiogenic growth factors, vascular endothelial growth factor (VEGF) plays a pivotal role in angiogenesis.

Clinical Perspective p 1784

VEGF specifically stimulates the proliferation and migration of endothelial cells in vitro and triggers a series of events that induce new blood vessel growth in vivo. It has been reported that VEGF expression is upregulated in myocardium and in serum of peripheral blood after myocardial ischemia. The administration of recombinant VEGF protein or VEGF gene into ischemic myocardium has been shown to enhance collateral vessel flow and improve cardiac function, although many reports suggest that the effects of VEGF are beneficial, the safety of this therapy is still a major concern. High doses of VEGF may lead to the formation of hemangiomata, and the diffusion of VEGF may cause undesirable side effects. Therefore, the identification of a specific target of VEGF in injured myocardium may offer an optimal therapy for myocardial ischemia.

The main components of cardiac extracellular matrix are type I and type III collagen, and ~85% of total collagen is type I. Type I collagen is increased at both the mRNA and protein levels in the infarct and noninfarct zones after myocardial infarction. Thus, collagen I could be a potential target for VEGF to be retained and enriched in the infarcted myocardium, enhancing the efficacy of VEGF in inducing revascularization for the alleviation of cardiac dysfunction. In a previous study, de Souza et al demonstrated that a heptapeptide (TKKTLRT) could bind specifically to type I collagen. Taking advantage of this heptapeptide, we produced a fusion protein that consists of VEGF and the collagen-binding domain (CBD-VEGF). We demonstrated that the...
fusion protein could bind stably to collagen I and maintain VEGF activity both in vitro and in vivo. In the rat acute myocardial infarction model, we found that CBD-VEGF could be retained and concentrated at the border zone of infarction to improve cardiac function.

Methods

Plasmid Construction and Protein Production

Full-length complementary DNA of human VEGF<sub>165</sub> was amplified by polymerase chain reaction from the complementary DNA of human breast tumor cell line MCF-7. CBD-VEGF was constructed by linking a sequence that encodes the collagen-binding domain (TKKTLRT) with VEGF<sub>165</sub> complementary DNA. Native VEGF or CBD-VEGF, respectively, was inserted into pET-28a (Novagen, Madison, Wis). The plasmid was transformed into the BL21 strain of Escherichia coli. Protein was induced with 1 mmol/L isopropyl β-d-thiogalactopyranoside for 5 hours. After it was refolded, the protein containing 6× His tag was purified by nickel chelate chromatography and HiTrap heparin HP columns (GE Healthcare, Chalfont St Giles, United Kingdom).

Collagen-Binding Assay

Type I collagen prepared from rat tail tendon as described previously was added to 96-well plates. The plates were blocked with 5% casein for 2 hours. After they were washed with PBS supplemented with 0.05% Tween-20 and 0.5% BSA, they were incubated with 100 μL/well of serial dilutions of VEGF or CBD-VEGF in PBS plus 1% BSA at 37°C for 2 hours. The remaining proteins on collagen were detected with an anti-VEGF monoclonal antibody (1:500, Santa Cruz Biotecnology, Santa Cruz, Calif).

Biological Activity of VEGF and CBD-VEGF

Human umbilical vein endothelial cells (HUVECs) were cultured in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum and 10% horse serum. Next, cells were seeded at a density of 5×10<sup>4</sup> cells/well with Iscove’s modified Dulbecco’s medium/10% fetal bovine serum in 48-well plates. After 8 hours, these cells were stimulated with commercial recombinant human VEGF (rhVEGF; PeproTech, Rocky Hill, NJ); native VEGF, or CBD-VEGF at serial concentration for 3 days. Cell growth–promoting activity was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. According to the standard curve, we translated absorbance values into number of cells.

Growth Factor Activity on Collagen In Vitro

The type I collagen–coated 48-well plates were blocked with 5% casein and incubated with 100 μL/well of serial dilutions of VEGF or CBD-VEGF in PBS plus 1% BSA at 37°C for 2 hours. The plates were washed with PBS supplemented with 0.05% Tween-20 and 0.5% BSA several times. Afterward, HUVECs were seeded and cultured for 3 days. Cell growth–promoting activity was detected by MTT assay.

Subcutaneous Implantation of Collagen Membranes

Collagen membranes were provided by Zhenghai Biotecnology Inc (Shandong, China). VEGF (0.5 nmol [10.32 μg]) or CBD-VEGF (0.5 nmol [11.12 μg]) was loaded onto each collagen membrane (1 cm×1 cm×1 mm). Collagen membranes loaded with PBS were used as the control. All experimental procedures were conducted in accordance with local guidelines on the ethical use of animals and the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 80-23, revised 1996). Male Sprague-Dawley rats (weight 180 to 200 g) were anesthetized with pentobarbital sodium (40 mg/kg). The collagen membrane loaded with proteins was implanted subcutaneously.

Myocardial Infarction Followed by Protein Injection

Briefly, male Sprague-Dawley rats (weight 180 to 200 g) were anesthetized and ventilated mechanically. After a thoracotomy was performed at the left fourth intercostal space, the left anterior descending coronary artery was ligated with a 6-0 silk suture below the tip of the left atrial appendage. Immediately after left anterior descending artery ligation, a total of 100 μL of saline, VEGF (0.5 nmol [10.32 μg]), or CBD-VEGF (0.5 nmol [11.12 μg]) was injected into 5 sites in the border zone.

Growth Factor Binding Ability in the Infarcted Heart

Three hours after administration of saline, VEGF, or CBD-VEGF, 1% Evans Blue dye (3 mL/kg, Sigma, St Louis, Mo) was injected into the right ventricle and allowed to circulate for ~20 seconds to define the nonperfused myocardium. After that, animals were euthanized by an intravenous overdose of ketamine. The infarct zone (with no Evans Blue staining) and the 1-mm rim surrounding the infarct zone (defined as the peri-infarct zone) were cut away from each slice and frozen immediately in liquid nitrogen. Next, protein was extracted for Western blot analysis. Anti-polyhistidine monoclonal antibody (1:3000, Sigma) was used to distinguish exogenous proteins from endogenous proteins.

Serum VEGF and CBD-VEGF Levels

After saline, VEGF, or CBD-VEGF was injected into acute myocardial infarction rats for 3 and 6 hours, the serum was collected and measured with a human VEGF ELISA kit (ZSGB-BIO, Beijing, China).

Echocardiography

Four weeks after left anterior descending artery ligation, echocardiography was performed with a 10-MHz linear transducer and a cardiovascular ultrasound system (SONOS model 5500, Hewlett-Packard, Palo Alto, Calif). Parasternal long-axis and short-axis views were used to obtain 2-dimensional and M-mode images. Left ventricular (LV) end-systolic dimension (LVDs) and LV end-diastolic dimension (LVDd) were measured in the M-mode tracings at the midpapillary level. Percentage LV fractional shortening (%FS) was calculated as %FS = (LVDd−LVDs)/LVDs×100 (%). LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) were determined as LVEDV = 7.0×LVDd<sup>2</sup>/2.4+LVDd and LVESV = 7.0×LVDs<sup>2</sup>/2.4+LVDs, as described previously.14 Percentage LV ejection fraction (EF%) was calculated as EF% = (LVEDV−LVESV)/LVEDV×100 (%).

Histological Analysis

After hemodynamic measurements, hearts were excised rapidly and sliced into 3 segments from apex to base. Each segment was fixed in 4% paraformaldehyde for 48 hours and embedded in paraffin. Sections (5 μm) were cut from each segment and stained with hematoxylin and eosin and with Masson trichrome. The Masson trichrome–stained sections from the middle segment were analyzed for scar size and infarct wall thickness by Image-Pro Plus (version 5.0).

Serial sections were immunolabeled with anti-von Willebrand factor antibody (1:150, Chemicon, Temecula, Calif). The number of capillary vessels was counted from at least 6 randomly selected fields in the border zone of infarction under a magnification of ×400.
Statistical Analysis

All data are expressed as mean±SEM. Statistics were calculated with SPSS computer software for Windows (version 13.0, SPSS Inc, Chicago, Ill). Student t test was used to compare collagen-binding ability in vitro and in vivo and the biological activity on collagen between VEGF and CBD-VEGF. One-way ANOVA, followed by a Bonferroni-Dunn test, was used to compare HUVEC growth-promoting activity, neovascularization in collagen membrane and border zone, echocardiography results, hemodynamics, scar size, and infarct wall thickness between control, VEGF, and CBD-VEGF. Welch ANOVA, followed by the Dunnett T3 test, was used to compare the serum VEGF level between control, VEGF, and CBD-VEGF. Results were considered to represent significant differences at *P<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

CBD-VEGF Bound Specifically to Type I Collagen In Vitro

The in vitro collagen-binding activities of VEGF and CBD-VEGF were measured by ELISA. As shown in Figure 1A, the binding curves of VEGF and CBD-VEGF were different. At a concentration range of 0.12 to 7.5 μmol/L, the optical density at 405 nm (OD_{405}) in CBD-VEGF was significantly higher than that of VEGF, which indicates that more CBD-VEGF bound to type I collagen than VEGF. Using the binding curve, the dissociation constant (K_d) value of VEGF or CBD-VEGF binding to collagen (0.1 mg) was calculated by Scatchard analysis. The slope of the resulting straight line equals $1/K_d$ (Figure 1B). The $K_d$ value for the binding of VEGF or CBD-VEGF to 0.1 mg of collagen was calculated as 1.36 and 0.43 μmol/L, respectively. Because a lower $K_d$ represents a higher affinity to collagen, CBD-VEGF bound more specifically to collagen.

CBD-VEGF Promoted HUVEC Growth In Vitro

To test the biological activities of CBD-VEGF and VEGF, HUVECs were used. The proliferation-stimulating activity of CBD-VEGF and VEGF on HUVECs was examined and compared with commercial VEGF (control). As shown in Figure 2A, the dose-response curves of CBD-VEGF, native VEGF, and commercial VEGF were similar. No significant
differences of activity were found among them in each concentration. Thus, growth factor activity of CBD-VEGF was not affected by fusion with CBD peptide.

CBD-VEGF Stimulated HUVEC Proliferation Through Collagen Binding In Vitro
We examined whether CBD-VEGF retained its biological activity after it was bound to collagen. Collagen-coated 48-well plates were incubated with VEGF or CBD-VEGF; the plates were washed, and HUVECs were cultured on the plates. At a concentration range of 0.17 to 5.51 μmol/L, the proliferation ability of HUVECs cultured on CBD-VEGF–loaded collagen was higher than that of VEGF-loaded collagen (Figure 2B). Thus, CBD-VEGF retained its biological activity after it was bound to collagen in vitro.

CBD-VEGF Induced Blood Vessel Formation in Collagen Membranes After Subcutaneous Implantation
To systematically evaluate the function of CBD-VEGF in vivo, collagen membranes loaded with VEGF or CBD-VEGF were implanted subcutaneously in rats. After 14 days, the implants were retrieved and processed for histological analysis. As shown in Figure 3A, apparent vascularization was observed on collagen membranes in the VEGF and CBD-VEGF groups. The CBD-VEGF group showed more obvious vascularization. Immunohistochemistry (Figure 3B through 3E) demonstrated that very few blood vessels were present in the collagen membranes treated with PBS (2.14±0.37). Native VEGF induced an increase in blood vessel density compared with PBS (4.96±0.35; P<0.05), whereas CBD-VEGF stimulated much more blood vessel formation than native VEGF (10.16±0.86; P<0.01). Thus, CBD-VEGF induced neovascularization in collagen membranes more effectively in vivo.

CBD-VEGF Was Retained in the Infarct Border Zone
We evaluated whether CBD-VEGF could be retained in the infarct border zone. CBD-VEGF or VEGF was injected into 5 sites in the border zone that surrounded the infarct (Figure 4A); 3 hours later, cardiac proteins were extracted and assessed by Western blot. An anti-polyhistidine antibody was used to detect exogenous VEGF in the border zone. As shown in Figure 4B and 4C, the level of exogenous CBD-VEGF was significantly higher than that of VEGF, which indicates that CBD-VEGF was retained and enriched in the infarct border zone.

To confirm whether VEGF or CBD-VEGF was diffused into the circulation, we measured serum VEGF and CBD-VEGF levels. Saline, CBD-VEGF, or VEGF was injected into infarcted hearts after left anterior descending coronary artery ligation; 3 and 6 hours later, serum from peripheral blood was collected and assessed by ELISA. As shown in Figure 4D, serum VEGF levels were higher than both control and serum CBD-VEGF levels after 3 hours. After 6 hours, serum VEGF levels were also higher than control, which meant the native VEGF had diffused into the circulation. Thus, much more CBD-VEGF than VEGF was retained at the infarct border zone; conversely, much more VEGF than CBD-VEGF was diffused.

CBD-VEGF Improved Cardiac Function After Myocardial Infarction
Four weeks after myocardial infarction, cardiac function was measured by echocardiography and hemodynamics.
Data are presented as mean ± SEM. *P < 0.05, **P < 0.01.

Figure 4. Binding ability of VEGF and CBD-VEGF to the infarcted heart. A, After ligation, saline, VEGF, or CBD-VEGF was injected into 5 regions in the border zone surrounding the infarct. Black dots indicate injection sites. B, Three hours after injection, exogenous protein levels were detected by Western blot with anti-polyhistidine antibody. β-Actin was used as internal control. C, Quantitation of protein bands (n = 4 in each group). D, Exogenous protein levels in serum were detected 3 and 6 hours after injection (n = 6 in each group). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01.

diography findings (Table 1) showed that both LV fractional shortening and LV ejection fraction in the CBD-VEGF group were markedly higher than in the control group. No statistically significant difference was found between the VEGF group and control group in fractional shortening or ejection fraction. No significant differences were found in LVd, LVDs, interventricular septal diastolic thickness, or LV posterior wall thickness among the control, VEGF, and CBD-VEGF groups.

Hemodynamic findings (Table 2) showed a significant improvement in both maximum and minimum dP/dT in the CBD-VEGF group compared with the control group, which suggests that both systolic and diastolic function were preserved in the CBD-VEGF group. No significant differences were found in LV systolic pressure, LV end-diastolic pressure, or heart rate among the 3 groups.

The scar size and infarct wall thickness were determined by Masson trichrome staining (Figure 5). The percentage of scar size in the CBD-VEGF group (20.01 ± 6.16%) was significantly reduced compared with the control group (45.78 ± 7.15%; P < 0.05), and infarct wall thickness in the CBD-VEGF group (2.68 ± 0.37 mm) was significantly greater than in the control group (1.26 ± 0.22 mm; P < 0.05). Taken together, this demonstrated that CBD-VEGF enhanced cardiac function and improved cardiac morphology after myocardial infarction.

CBD-VEGF Induced Angiogenesis After Myocardial Infarction
To detect the angiogenesis effect of CBD-VEGF, we measured capillaries in the border zone of the infarction by immunohistochemical staining for von Willebrand factor (Figure 6). Capillary density was significantly higher in the CBD-VEGF group than in the VEGF and control groups (236.05 ± 15.74, 179.31 ± 11.43, and 146.68 ± 9.67, respectively; P < 0.01 versus VEGF, P < 0.01 versus control). Capillary density in the VEGF group was also significantly higher than in the control group (P < 0.05). Thus, CBD-VEGF induced angiogenesis after myocardial infarction much more effectively.

Discussion
We demonstrated that CBD-VEGF shares the characteristics of both specific binding to collagen and stimulation of endothelial cell proliferation in vitro. CBD-VEGF induced significant blood vessel formation in collagen membranes after subcutaneous implantation. Injection of CBD-VEGF reduced scar size and improved cardiac function in the rat acute myocardial infarction model, because immobilization of CBD-VEGF on cardiac collagen around the infarct zone resulted in a high local concentration and prolonged the biological effect. In addition, CBD-VEGF induced a greater amount of mature capillary formation than VEGF or control, which may provide a possible mechanism for tissue repair.

Previous clinical trials of VEGF in acute myocardial infarction have focused on virus-mediated or naked plasmid containing VEGF complementary DNA transfection. These were reported to significantly reduce anginal class and improve end points (phase 1/2 trial)15 and to improve perfusion and wall motion in the area of vector administration.
Fewer clinical trials of VEGF protein have been reported, with 2 impressive ones being the study by Hendel et al\textsuperscript{17} and the VIVA (Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis) Trial.\textsuperscript{18} In the phase 1 trial by Hendel et al,\textsuperscript{17} rhVEGF improved myocardial perfusion at rest and provided evidence of a dose-dependent effect (high-dose rhVEGF resulted in significant improvement).\textsuperscript{17} However, the VIVA Trial revealed that rhVEGF offered no improvement beyond placebo by day 60, although high-dose rhVEGF resulted in better improvement in angina and favorable trends in exercise treadmill test time and angina frequency by day 120.\textsuperscript{18} According to these trials, we may speculate that an effective dose of VEGF in myocardium is crucial for effective therapy.

To maintain VEGF concentration, multiple injections may be needed; however, this would increase both the cost and surgical risk of this potential therapy. In addition, excessive VEGF at injection sites and the diffusion of VEGF may cause possible adverse effects. It has been reported that unregulated continuous expression of VEGF leads to the formation of angioma at the site of injection.\textsuperscript{8,19} A high level of circulating VEGF in acute myocardial infarction induces an acute cor pulmonale and results in increased mortality.\textsuperscript{20} Thus, the delicate control of VEGF protein both in dosage and localization is important to enhance its local therapeutic efficiency and decrease its possible adverse effects.

Many groups are working on localizing and sustaining VEGF proteins at the sites of injury to treat tissue ischemia.

### Table 2. Evaluation of Cardiac Function by Hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>LVSP, mm Hg</th>
<th>LVEDP, mm Hg</th>
<th>HR, bpm</th>
<th>Maximum dP/dt, mm Hg/s</th>
<th>Minimum dP/dt, mm Hg/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>136.6±4.4</td>
<td>15.3±2.0</td>
<td>439±13</td>
<td>7223±331</td>
<td>−5762±298</td>
</tr>
<tr>
<td>VEGF</td>
<td>138.7±4.3</td>
<td>12.0±2.8</td>
<td>438±18</td>
<td>7833±356</td>
<td>−6621±439</td>
</tr>
<tr>
<td>CBD-VEGF</td>
<td>151.0±3.4</td>
<td>8.0±0.9</td>
<td>470±15</td>
<td>9014±502*</td>
<td>−7909±542†</td>
</tr>
</tbody>
</table>

LVSP indicates LV systolic pressure; LVEDP, LV end-diastolic pressure; and HR, heart rate.

Data are mean±SEM. n=9 in each group.

\*P<0.05 vs control; †P<0.01 vs control.

### Figure 5.

Histological analysis of hearts 4 weeks after myocardial infarction. Heart sections were stained with Masson trichrome. A, Control group. B, VEGF group. C, CBD-VEGF group. D, Percentage of infarct area in the whole LV area. E, Wall thickness of infarct area. n=7 in each group. Data are presented as mean±SEM. \*P<0.05. Scale bar=1 mm.
Both injectable alginate hydrogel and poly(lactide-co-glycolide) have been used as vehicles to deliver VEGF to ischemic hindlimbs. Moreover, the temporal and spatial release of VEGF from hydrogel in the ligated area enhances blood flow and prevents necrosis in limbs. Hao et al. have demonstrated that delivery of recombinant VEGF and platelet-derived growth factor from alginate hydrogel promotes angiogenesis and improves cardiac function in a myocardial infarction model. To take advantage of fibronectin binding to growth factors, fibronectin-CBD-VEGF specifically targets the collagen matrix and stimulates both endothelial cells and endothelial progenitor cells (EPCs) in situ, which provides a vascular regeneration niche.

It is known that collagen I and collagen III constitute the bulk of the cardiac extracellular matrix, and ~85% of total collagen is type I. After myocardial infarction, the activity of matrix metalloproteinases (MMPs) is highly increased, which induces collagen degradation in the infarct zone. Subsequently, collagen synthesis is activated and sustained for a long time, whereas early collagen degradation is short-lived and suppressed in the fibrogenic phase. During this stage, both collagen type I and type III are increased in the infarct zone, but the increase in collagen type III is greater. In later stages, the collagen type I/III ratio is increased, which results in great resistance to distension. In the early stage, although MMP activity is highly increased in the infarct zone, both expression and activity of MMPs are increased modestly in the border and remote zones. Thus, MMP-induced collagen degradation in the border zone may be milder, which results in better preservation of collagen type I. During the healing stage, collagen type I is increased in both infarcted and noninfarcted myocardium, and collagen I may also be increased in the border zone. Therefore, type I collagen may still be a potential target for VEGF to be concentrated in the infarct border zone. In the present study, the collagen-binding peptide TKKTTLRT was fused to VEGF to specifically target CBD-VEGF to type I collagen. The TKKTTLRT peptide was derived from a nucleotide sequence that was complementary to that which codes for the interstitial collagen domain, which is attacked by collagenase. Type I collagen binds specifically and quantitatively to this peptide. CBD-VEGF was able to bind specifically not only to the collagen gel and collagen membrane but also to native collagen in the cardiac extracellular matrix.

Delivery of CBD-VEGF clearly alleviated cardiac dysfunction and reduced scar size, which might be closely related to its effect in the acute and healing phase. It is known that early remodeling after myocardial infarction is initially caused by myocyte necrosis. In the present study, CBD-VEGF was delivered into the peri-infarct zone immediately after infarction and retained an adequate concentration over time. In the acute phase, CBD-VEGF reduced endothelial and myocardial apoptosis and improved cardiomyocyte viability in the infarct border zone after ischemia. During the healing phase, CBD-VEGF enhanced blood vessel growth and perfusion in ischemic myocardium, which may subsequently attenuate infarct extension as a result of secondary myocyte loss that occurs in the infarct border zone. After myocardial infarction, the infarct bed capillary network is inadequate to handle the greater demands of the hypertrophied but viable myocardium. The relative lack of oxygen and nutrients might be an important causative factor in the death of otherwise viable myocardium, resulting in progressive infarct extension and fibrous replacement. Thus, CBD-VEGF may induce angiogenesis and augment the development of collateral vessels that decrease apoptosis of ischemic myocytes in the peri-infarct region and increase long-term survival of viable myocardium. During the posthealing period, the cascade of increased myocardial blood flow and angiogenesis triggered by CBD-VEGF may be sustained, which would result in improvement of cardiac function. Meanwhile, native VEGF was less efficient in preserving cardic function than CBD-VEGF, because much of the VEGF protein was washed away by body fluids, whereas CBD-VEGF was retained in the border zone. The high binding ability of CBD-VEGF to the infarcted myocardium increased the retention of VEGF and thus improved its therapeutic efficiency.

VEGF is a key regulator of blood vessel formation during both angiogenesis (new blood vessels sprouting from preexisting blood vessels) and vasculogenesis (new blood vessels deriving from EPCs). Figure 4 demonstrates a high CBD-VEGF level in the infarct border zone and less diffusion of CBD-VEGF into the circulation. Because the angiogenic effect of VEGF is strongly dependent on its local concentration, the CBD-VEGF used in the present study may be favorable for the induction of angiogenesis. However, angiogenesis alone may not be sufficient therapy for myocardial ischemia, and vasculogenesis may be still required for better recovery. Previous studies have demonstrated that bone marrow–derived EPCs, with stimulation of some cytokines or tissue ischemia, home to the sites of ischemia and directly incorporate into new blood vessels. In addition, the recruitment of EPCs may have beneficial effects on animal models of ischemic disease. In the meantime, VEGF mobilizes EPCs from bone marrow to peripheral blood, and the
plasma levels of VEGF correlate positively with the number of CD34-positive mononuclear cells, which are putative precursor of EPCs. In the present study, low levels of CBD-VEGF in the circulation (Figure 4D) may have caused less recruitment of EPCs. Similar work on collagen-targeting VEGF has demonstrated that local delivery of fibronectin CBD-VEGF121 does not promote EPC mobilization, whereas VEGF121 does. Thus, the CBD-VEGF used in the present study may not significantly induce vasculogenesis. Because some cytokines have shown high efficiency for EPC mobilization and have contributed to neovascularization of ischemic tissues, the combined application of CBD-VEGF with a cytokine (such as granulocyte colony-stimulating factor) could be more beneficial for therapy of myocardial ischemia.

The present study has certain limitations. First, 2-dimensional guided M-mode echocardiography was used to evaluate cardiac function. Although these measurements provided information on LV dimensions and fractional shortening at the mid–papillary muscle level, 2-dimensional echocardiography in multiple sections from different views and 3-dimensional reconstruction could be used to obtain more accurate parameters. Second, regional blood flow was not measured by a radioactive microsphere technique or with nuclear perfusion imaging techniques; consequently, the functionality of vascularization was not determined.

Acknowledgments
We thank Dr Hongbing Zhang and Dr Bing Liu for their helpful comments on revising the manuscript. We thank Dr Di Xu for his technical assistance in echocardiography.

Sources of Funding
This work was supported by grants from the National Natural Science Foundation of China (30688002; 30603034), the Chinese Academy of Science (KSCX2-YW-R-133), and the Ministry of Science and Technology of China (2006CB943601).

Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Clinical trials of vascular endothelial growth factor (VEGF) gene therapy have proven that VEGF has beneficial effects on ischemic heart disease; however, potential problems associated with the prolonged and excessive production of VEGF cannot be ignored. Clinical studies of VEGF protein revealed evidence of a dose-dependent effect (ie, high-dose VEGF protein resulted in a better therapeutic effect). Given the side effects of high-level VEGF in the circulation, an increase in the local VEGF level may represent a reasonable treatment for ischemic heart disease. In the present study, we investigated the angiogenic effect of a fusion protein composed of VEGF and a collagen-binding domain (CBD-VEGF) and its therapeutic effects in a rat acute myocardial infarction model. The results demonstrated that CBD-VEGF induced much more blood vessel formation than native VEGF in collagen membranes implanted subcutaneously. Compared with native VEGF, CBD-VEGF maintained a higher level in extracellular matrix and a lower level in circulation, and it preserved heart function, reduced scar size, and increased capillary vessels after injection into the border zone of acute myocardial infarction in rats. Although the latent side effects and precise role of CBD-VEGF during healing must be investigated further, our results may suggest a novel therapeutic approach for patients with ischemic heart disease.